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# MODULATION OF IRON TRANSPORT, METABOLISM AND REACTIVE OXYGEN STATUS BY QUERCETIN-IRON COMPLEXES *IN VITRO*

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**Abbreviations:** **C3G** (Cyanidin-3-O-glucoside), **FER** (Ferritin), **GPX4** (Glutathione peroxidase 4), **IBD** (Inflammatory Bowel Disease), **IRP2** (Iron Regulatory Protein 2), **ITC** (Isothermal Titration Microcalorimetery), **LIP** (Labile Iron Pool), **M** (Media), **Q** (Quercetin), **ROS** (Reactive Oxygen Species), **TfR1** (Transferrin Receptor 1).

**Key Words:** Chelation; Iron; Quercetin; Reactive Oxygen Species.

## ABSTRACT

**SCOPE:** Excess free-iron is detrimental to health through its ability to participate in free radical generation and amplification of oncogenic pathways. The study aims were to identify polyphenols with iron-chelating potential.

**METHODS AND RESULTS:** Of four polyphenols tested quercetin demonstrated potent iron binding with the physiological outcome dictated by the location of interaction. In the presence of extracellular iron and quercetin, ferritin expression and cellular iron concentrations decreased suggesting the resulting quercetin-iron complex is not internalised. However, in the relative absence of extracellular iron, quercetin becomes internalised and complexes with both intracellular iron, and iron which subsequently becomes absorbed as indicated by increased cellular $^{59}Fe$ post pre-culture with quercetin. This increased intracellular iron complexed to quercetin does not associate with the labile iron pool and cells behave as though they are iron-deficient (increased transferrin receptor-1 and iron regulatory protein-2 expression and low ferritin expression). Additionally, a suppression in reactive oxygen species was observed.

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**CONCLUSION:** Quercetin, an exogenous iron chelator, is able to render the cell functionally iron-deficient which not only provides a therapeutic platform for chelating excess free luminal iron but also may be of use in limiting processes like cancer-cell growth, inflammation and bacterial infections, which all require iron.



## INTRODUCTION

Whilst iron is essential for life, excess free-iron is linked to a range of disorders including neurodegenerative diseases, cardiovascular disease and cancer.[1-3] The mechanism of free-iron (non-ligated/ non-chelated iron) toxicity is attributed to its ability to react with peroxide and superoxide under physiological conditions and catalyse the formation of the pro-inflammatory hydroxyl radical.[4] Thus, unsurprisingly, high levels of free-iron in the gastro-intestinal tract is associated with disease.[5-7] Since 0.7 – 22.9 % of ingested dietary iron is absorbed at the small bowel for nutrition, the remaining iron resides within the lumen of the bowel for hours to days.[8] As a consequence, this 'luminal pool' of iron has been identified as a toxic agent implicated in intestinal disease.[9, 10] Most notably two murine studies have shown that removal of dietary iron from models of inflammatory bowel disease (IBD) and intestinal cancer resulted in a suppression of disease phenotype, whilst consumption of excess dietary iron exacerbated the conditions.[5, 9] However, how this pool of iron is involved in disease is less clear, though it seems likely that iron-mediated reactive oxygen species (ROS) generation is a contributing factor.[11-14] Previous reports have also implicated iron in the Wnt signalling pathway, where iron has been demonstrated to amplify Wnt signalling and iron chelators inhibit.[15, 16] Thus, the use of a compound that neutralises this pool of free-iron, through iron chelation, represents a therapeutic platform in the prevention of these gastro-intestinal diseases. If such compounds elicited additional, intracellular iron modulation effects, such a strategy could also be useful in treating a number of other pathologies (including cancer, hereditary

haemochromatosis and malaria) since the processes which underpin these conditions (cell growth, inflammation and infection) are reliant upon free-iron.[1, 17, 18]

There are a number of dietary agents including polyphenolic compounds which are able to either bind iron and/or have anti-oxidant properties.[19, 20] Polyphenols found in fruits and vegetables possibly have multiple actions in preventing disease by; i) chelating iron to stop the catalysis of ROS, ii) binding iron to supress iron-mediated processes and iii) scavenging any ROS present in a direct anti-oxidant manner.[21-23] Polyphenolic compounds have a range of chemical moieties, and previous literature suggests that iron chelation is likely to take place when i) ortho-dihydroxyl groups; ii) C5-OH and/or C3-OH moieties in conjunction with a C4 keto-group; and iii) a large number of OH groups are present.[24] In addition, there is a plethora of evidence highlighting their usefulness as anti-oxidants.[21, 25-27] Whether this radical-scavenging activity would occur within colonic cells is unknown and their impact on iron metabolism within colonocytes is also yet to be determined. Polyphenolic compounds have been documented to be unstable and labile towards microbial metabolism within the gastro-intestinal tract, hence any compounds identified with iron chelating properties would need to be administered as a supplement, and targeted solely to the colon to ensure bioactivity and avoid interfering with small bowel iron absorption.[28]

Thus the aim of this study was to examine four polyphenols all found in the human diet (quercetin, rutin, cyanidin-3-O-glucoside and catechin) with documented evidence of presence within the colon[29] and determine their iron binding capacity, impact on colonocyte iron metabolism and effect on iron mediated free radical generation. This experimental approach will provide insight into the selection of a polyphenol that could be used to combat free-iron mediated disease, including gastro-intestinal disease.

## MATERIALS AND METHODS

### Isothermal Titration Microcalorimetry

Quercetin, rutin, and catechin were dissolved in Dimethyl Sulphoxide (DMSO), whilst cyanidin-3-O-glucoside was reconstituted in Deionised (DI) $H_2O$ producing a 0.05 M stock solution. Polyphenols (0.05 mM) were diluted in potassium phosphate buffer (0.1 M, pH = 7). Both iron chloride and ferrous sulphate solutions (0.5 mM) were prepared by dissolving $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ in aqueous HCl (0.1 M) respectively. Measurements were performed on a VPITC MicroCalorimeter and were analysed using MicroCal LLC ITC/Origin software package.[30]

**Cell culture**

RKO cells (ATCC), an established colon carcinoma derived cell line of epithelial origin, were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with Foetal Calf Serum (10 % v/v), penicillin (100 U ml $^{-1}$) and streptomycin (0.1 mg mL $^{-1}$) and were routinely checked for mycoplasma infection. The iron within the FCS supplemented media is bound to transferrin and other iron-binding proteins, and thus there is negligible free-iron available. Cells were seeded into either 6, 12 or 96 well plates at 1 X $10^5$ cells mL$^{-1}$ (2 mL, 1 mL and 100 µL for each well type respectively) and cultured in growth medium for 24 hours prior to experimentation.

In order to manipulate polyphenol and iron culturing regimens, two variations were performed:

i) ***Iron and polyphenol simultaneously present within the culture media:*** RKO cells were challenged with iron supplemented growth media containing iron ($FeSO_4 \cdot 7H_2O$, 100 µM) and sodium ascorbate (5 mM) with or without polyphenol supplementation (0 - 200 µM) simultaneously.

ii) ***Cells were pre-cultured with polyphenol prior to culture with iron:*** RKO cells were co-cultured with polyphenol (200 µM) for 12 hours before this

media was removed, cells washed, and then co-cultured with iron ($FeSO_4 \cdot 7H_2O$, 100 μM) and sodium ascorbate (5 mM) for 24 hours.

After these subsequent incubation periods, cells were washed in Phosphate Buffered Saline (PBS) and lysed for further analysis according to the specific experiment. The use of sodium ascorbate with iron in a 50:1 iron: ascorbate ratio ensures that all supplemented iron is in a soluble 'free' state and not precipitated as aggregates.

**Western blotting**

Cells were lysed in RIPA lysis solution (4-Nonylphenyl poly(ethylene glycol) (1 % w/v), sodium deoxycholate (0.5 % w/v), sodium dodecyl sulphate (0.1 % w/v)) on ice. Cell lysates were sonicated for 5 sec and protein concentrations determined using a protein assay (Pierce, BCA assay). Cell lysates were then subject to Western blotting as previously described.[31] Primary antibodies used were Ferritin light-chain (Abcam AB69090, 1:5000 dilution), β-actin (Abcam AB8226, 1:5,000 dilution), TfR1 (Invitrogen, H68.4, 1:1000), IRP2 (Cell Signalling Technology, #37135, 1:200) and GPX4 (Abcam, AB125066, 1:1000).

**$^{59}$Fe radioisotope studies**

Cells were seeded into 12 well plates and incubated for 24 hours before growth medium was replaced with conditioned media as described above (for radioisotope studies the iron supplemented growth media was spiked with $^{59}FeCl_3$ to reach ca. 10,000 counts per minute (CPM) per well.). After this incubation period, cells were washed in Versene (0.2 g $L^{-1}$ in PBS) and lysed in HEPES-saline lysis buffer (150 μL, 10 mM, pH 7.4, NaCl (0.9 % w/v)). Radioactivity was counted by mixing cell lysate sample with scintillation fluid (PerkinElmer) and radiation CPM were normalised to protein concentration as determined using a protein assay.

**Reactive oxygen species assay**

Two variations of this experiment were carried out as follows

*i)* ***Iron and Polyphenol simultaneously present within the media:*** RKO cells (1 X $10^4$) were incubated for 24 hours with standard growth medium in 96 well plates. Cells were then washed with PBS after which the ROS ligand (Cm-H2DCFDA, Lifetechnologies, 5 μM, 100 μL) was added for 1 hour. A baseline reading was taken (t=0) at λ = 485/535 nm. The ROS ligand conditioned media was then removed, and cells washed with PBS before the addition of the quercetin (20 μM) containing media with or without iron. Samples were analysed at 3, 12 and 24 hours.

*ii)* ***Iron pre-culture followed by quercetin incubation:*** RKO cells (1 X $10^4$) were incubated for 12 hours with standard growth medium in 96 well plates before the addition of iron supplemented media ($FeSO_4 \cdot 7H_2O$,100 μM) with sodium ascorbate (5 mM) and cultured for a further 12 hours. The iron supplemented medium was then removed, cells washed and ROS ligand added to cells for 1 hour. Cells were then treated as above in (i).

In each experiment, a control plate containing polyphenol only with no cells was prepared such that the absorption arising from the polyphenols could be subtracted.

**Labile Iron Pool Fluorescence-activated cell sorting**

RKO cells (1 X $10^4$) were incubated for 24 hours with standard growth medium in 6 well plates. Prior to experimentation, cells were loaded with the fluorescent dye Calcein AM (Corning). Cells were cultured with Calcein AM (0.0625 μM) for 15 mins at 37 °C in PBS. Calcein-AM was removed from the cells, washed X2 with PBS and subsequently co-cultured with either quercetin (200 μM) or $FeSO_4 \cdot 7H_2O$ (100 μM) as described above. At the end of the culture period media was removed, cells washed X2 with PBS, trypsinised and

centrifuged (600 RPM, 5 min). The cell pellet was re-suspended in PBS and re-centrifuged. The cell pellet was then finally re-suspended in PBS (150 µL), and analysed for mean fluorescence intensity in FL-1 on an Accuri-C6 flow cytometer (BD).

### Data and statistical analysis

All experiments were performed in triplicate unless otherwise stated. ITC data fitting was performed using the MicroCal LLC ITC/Origin software package according to the model of best fit for each data set.[30] Data were analysed by Student's *t*-test and analysis of variance where appropriate. Values are presented as the means of triplicates with error bars representing standard error of the mean (SEM).

## RESULTS

### Characterisation of iron binding to polyphenols as assessed by isothermal titration microcalorimetry

To probe the interaction of iron with the polyphenols Isothermal Titration Micro-Calorimetry (ITC) was employed under physiological conditions. Ferric iron or ferrous iron was titrated into polyphenol at a temperature of 37 °C. Isotherms were fitted using models of host-guest interactions (Figure 1). Results demonstrate that quercetin binds both ferrous and ferric iron and the number of binding sites on quercetin was calculated to be N = 0.63 ± 0.0163 and 1.16 ± 0.0261 (for ferrous and ferric iron respectively), indicating one preferred iron binding site on quercetin (Figure 1A). Iron binding constants were estimated to be $K = 8.3 \times 10^5\ M^{-1}$ and $3.86 \times 10^6\ M^{-1}$ for ferrous and ferric iron binding respectively. Iron binding by rutin could only be demonstrated with ferrous iron with no evidence of ferric iron binding (Figure 1B). Isotherms for ferrous iron interactions with rutin indicated two binding sites with K values of $K = 3.2 \times 10^8\ M^{-1}$ and $K = 2.2 \times 10^4\ M^{-1}$. For both cyanidin-3-O-glucoside and catechin, no appreciable iron binding parameters could be obtained (Figure 1C-D). Table 1 summarises the iron binding properties found under these physiological conditions.

**Effect of polyphenol on cellular ferritin expression**

To assess effects of polyphenols on cellular iron metabolism, colonic epithelial (RKO) cells were challenged with iron in the presence or absence of polyphenols (quercetin, rutin, catechin and cyanidin-3-O-glucoside) at a range of concentrations (0-200 μM). In these experiments, iron and polyphenols were both simultaneously present in the media. After 24 hours, cellular ferritin protein expression was evaluated as a surrogate marker for intracellular iron concentrations by Western blotting (Figure 2). As expected, treating cells with iron in all cases resulted in significant increases in ferritin protein expression ($p < 0.001$ for all) (Figure 2A-D). This iron mediated induction in ferritin protein expression was significantly suppressed by 51 % when cells were co-cultured with quercetin at 200 μM compared to the iron only control ($p < 0.05$) (Figure 2A). Similarly co-culture with rutin at 200 μM also significantly suppressed the iron mediated ferritin response by 54 % ($p < 0.05$) (Figure 2B). In support of the isothermal titration microcalorimetry results the polyphenols cyanidin-3-O-glucoside and catechin had no effect on the iron mediated induction in ferritin expression (Figure 2C-D), since they demonstrated no iron binding ability under these conditions.

**Effect of quercetin and rutin on intracellular iron**

To delineate whether the binding observed between the polyphenols quercetin and rutin impacted on intracellular iron levels in colonocytes, RKO cells were challenged with $^{59}Fe$ and as before cells cultured with or without quercetin and rutin (Figure 3). Analogous to above, iron and polyphenols were both simultaneously present in the media.

Co-culturing with quercetin at 200 μM and $^{59}Fe$ demonstrated a suppression in intracellular iron acquisition compared to cells challenged with iron alone (21 %, $p < 0.05$) (Figure 3A). However, co-culture with rutin at 200 μM resulted in a significant induction in cellular iron acquisition compared to cells cultured with iron alone (78 %, $p < 0.05$) (Figure 3B).

**Effect of quercetin pre-culture on cellular iron levels**

Co-culture of quercetin in the presence of iron reduces cellular iron concentrations and consequently results in a diminished iron-induced ferritin response presumably by binding iron extracellularly and inhibiting its cellular import. However, in subsequent experiments the effect quercetin might have on cellular iron metabolism when there is no extracellular iron present was examined. To address this, cells were pre-cultured with quercetin, cells washed and then subsequently co-cultured with iron. Exposure of colonocytes to quercetin and then subsequent co-culture with $^{59}$Fe increased the total intracellular $^{59}$Fe concentrations compared to cells that were a) pre-cultured with media alone followed by culturing with $^{59}$Fe (64 % $p < 0.0001$) and b) pre-cultured with iron followed by further culture with $^{59}$Fe (30 % $p < 0.01$) (Figure 4A).

Since pre-culture with quercetin induced total cellular iron we next investigated whether the labile iron pool was similarly impacted. Quercetin co-culture alone (with no exogenous iron) resulted in a marked suppression in the labile iron pool (125 %, $p < 0.005$) as assessed using FACS (Figure 4B). Pre-culture with quercetin followed by iron for 24 hours surprisingly resulted in a significant reduction in the labile pool relative to cells not exposed to a quercetin pre-culture (38 %, $p < 0.005$) (Figure 4C) and as expected, treating cells with iron alone significantly increased the labile iron pool relative to unchallenged cells (28 %, $p < 0.01$) (Figure 4C).

**Effect of quercetin on the expression of iron metabolism proteins by Western blotting**

Since pre-culture with quercetin elevated intracellular iron levels but suppressed the labile iron pool, the impact on cellular iron transport machinery was examined by Western blotting (Figure 5A-D).

*TfR1 Protein Expression*

Exposure to quercetin and subsequent co-culture with iron resulted in increased TfR1 protein expression compared to cells a) pre-cultured with media alone and then co-cultured with iron (49 %, $p < 0.01$) and b) pre-cultured with iron followed by further culture with iron (44 %, $p < 0.05$). Continuous culture with quercetin also resulted in increased TfR1 protein expression compared to unchallenged control cells (68 %, $p < 0.05$) (Figure 5A).

*Ferritin Protein Expression*

Exposure to quercetin and then subsequent co-culture with iron resulted in decreased ferritin protein expression compared to cells a) pre-cultured with media alone and then co-cultured with iron (44 %, $p < 0.05$) and b) pre-cultured with iron followed by further culture with iron (57 %, $p < 0.05$). Continuous culture with iron as expected resulted in a significant induction in ferritin protein expression compared to control cells cultured in media alone (112 %, $p < 0.001$) (Figure 5B)

*IRP2 Protein Expression*

Since both TfR1 and ferritin protein expression are known to be regulated by the iron regulatory protein 2 (IRP2) the expression of IRP2 was also examined. Exposure to quercetin and then subsequent co-culture with iron resulted in increased IRP2 protein expression compared to cells a) pre-cultured with media alone followed by iron (55 %, $p < 0.05$) and b) pre-cultured with iron followed by further culture with iron (28 %, $p < 0.05$). Continuous culture with quercetin also resulted in increased IRP2 protein expression compared to unchallenged control cells (137 %, $p < 0.0001$) (Figure 5C).

*GPX4 Protein Expression*

Glutathione Peroxidase 4 (GPX4) a known anti-oxidant enzyme associated with ROS was examined. Culture with quercetin and then subsequent co-culture with iron resulted in decreased GPX4 protein expression compared to cells a) pre-cultured with media alone followed by iron (33 %, $p < 0.005$) and b) pre-cultured with iron followed by further culture

with iron (17 %, $p < 0.05$). Continuous culture with quercetin also resulted in decreased GPX4 protein expression compared to unchallenged control cells (36 %, $p < 0.05$) (Figure 5D).

**Quercetin anti-oxidant effects**

To examine the antioxidant effects of quercetin and to address whether its antioxidant nature was attributed to its iron chelating potential, the cellular levels of ROS (Figure 6A-C) and expression of GPX4 were determined (Figure 6D) using both FACS and Western blotting respectively. RKO cells were co-cultured with quercetin or media only and intracellular ROS levels measured (Figure 6A). Quercetin significantly reduced intracellular ROS by 67, 45 and 33 % (at 3, 12 and 24 hours respectively) compared to control (all $p < 0.05$) (Figure 6A). When quercetin was co-cultured in the background of exogenous iron, quercetin was still able to suppress intracellular ROS concentration by 45 and 20 % (at 3 and 12 hour time points) compared to the iron supplemented media control ($p < 0.05$) (Figure 6B) (at 24 hours this reduction was 9 %, $p = 0.06$). When cells were pre-cultured with iron followed by culture with quercetin the level of ROS was dramatically reduced by 58, 44 and 44 % at 3, 12 and 24 hours respectively (all $p < 0.05$) compared to cells pre-cultured with iron alone (Figure 6C). These changes in detectable levels of cellular ROS were reflected in the expression of GPX4 (Figure 6D). Levels of GPX4 protein were significantly lower when comparing cells pre-cultured with iron and then challenged with quercetin compared to cells continually cultured with iron (49 %, $p < 0.01$)). In addition the levels of GPX4 protein were significantly suppressed when cells were cultured with quercetin compared to unchallenged cells (46 %, $p < 0.005$) (Figure 6D).

**DISCUSSION**

Previous reports in murine models have shown that the cytotoxic effects of excess dietary iron contribute to an inflammatory state within the lumen of the colon.[5, 9] The detrimental properties of free-iron can be attributed to its ability to catalyse the formation of reactive

oxygen species.[12] A recent report suggests that iron in conjugation with bile acids, K-vitamins and oxygen interact to induce an oncogenic effect in the colon, via the generation of free radicals.[11] Not only can free-iron take part in cytotoxic reactions within the lumen of the colon, free-iron has also been implicated in cancer-cell growth, inflammatory processes and fuelling bacterial infections.[1, 17, 18] A method of inhibiting this reactive nature of free-iron is through chelation. Polyphenolic compounds have the ability to both chelate iron and sequester ROS.[20, 32] For this reason, four polyphenolic dietary iron chelators were chosen (quercetin, rutin, cyanidin-3-O-glucoside and catechin), to assess their effects on colonocyte iron metabolism and levels of intracellular ROS.

Quercetin has previously been reported to bind iron, with the 1:2 Fe: quercetin complex found to be the most energetically stable and iron binding constants calculated to be between $K = 1 \times 10^6 – 10^{12}\ M^{-1}$ - $M^{-2}$ using spectroscopic methods.[33-35] These results are in agreement with the calorimetry data presented herein, which demonstrated that quercetin had iron binding constants of $K = 8.3 \times 10^5\ M^{-1}$ and $3.86 \times 10^6\ M^{-1}$ for ferrous and ferric iron respectively. With respect to rutin, the di-rutin iron complex has been shown to have iron binding constants of $K = 4 \times 10^{11} – 1 \times 10^{12}\ M^{-2}$ by spectroscopy, with results presented herein only establishing rutin-ferrous iron binding with a two-site binding mechanism; the calculated iron binding constants were $K = 3.2 \times 10^8\ M^{-1}$ and $K = 2.2 \times 10^4\ M^{-1}$ for the two sites. Interestingly, the calorimetry experiments identified that quercetin does indeed have two iron binding sites as predicted, with one site being the preferred site for coordination by iron. When this site is blocked as in the case for rutin, two other iron binding sites are consequently utilised for iron chelation as predicted by Khokhar *et al*.[24] For both cyanidin-3-O-glucoside and catechin no ferric or ferrous iron binding could be detected; neither of these compounds contain the preferred C3-keto group for iron chelation. This limited capacity for iron chelation was verified *in vitro* where neither of the compounds reduced iron induced ferritin protein expression.

There are a number of contrasting studies that have examined how quercetin can modulate cellular iron concentrations *in vitro*, acting as both an inhibitor of iron uptake and as an iron 'shuttle' (i.e. able to load cells with iron).[22,35,36] Experiments performed herein are able to explain why these opposing phenomena have been observed by altering the cellular exposure to 'free' (uncomplexed) quercetin. When cells were co-cultured with quercetin and iron simultaneously and using a Fe: quercetin molar concentration of 100:200 there would be no free quercetin available to enter the cell (since the 1:2 Fe: quercetin complex is most stable) and hence acts as an (extracellular) inhibitor of iron uptake. On the other hand, if any 'free' quercetin remained, it would be able to enter the cell and capture endogenous and any subsequent exogenous iron, trapping the iron and hence increasing intracellular iron concentrations.

When iron and polyphenol were simultaneously present in the culture media it was found that both quercetin and rutin do indeed inhibit iron-mediated ferritin protein expression and that quercetin statistically decreased intracellular $^{59}$Fe concentrations. These results support the ferritin protein expression findings, suggesting that quercetin is indeed chelating iron extracellularly and the subsequent iron-quercetin complex does not become internalised. Co-incubation of rutin alongside iron increased $^{59}$Fe compared to the iron only control. This infers that despite the fact that rutin is chelating iron, as evidenced by decreased ferritin expression, the chelated iron becomes intracellular. The subsequent rutin-iron complexes are unlikely to be contributing to the labile iron pool and thus not sensed by IRPs since a reduction in the iron-induced ferritin expression was observed.

It has previously been reported that quercetin iron complexes can readily efflux cells and experiments performed here do not rule out this possibility.[36] Such observations would suggest that quercetin and its complexes with iron are indeed cell-permeable. To address whether this was the case in colonocytes, the cellular exposure to quercetin was modified in such a way that quercetin-iron complexes could not form extracellularly and iron and quercetin would only interact intracellularly. The results of these experiments demonstrate

that the ability of quercetin to act as an extracellular or intracellular iron chelator is dependent on the location of the quercetin iron interaction. Specifically, when colonocytes were exposed to quercetin (with no extracellular iron) for 12 hours and then co-cultured with iron, intracellular $^{59}$Fe concentrations increased. This is in contrast to when quercetin and iron are able to interact extracellularly, where decreased intracellular iron concentrations were observed. Co-culture of cells with quercetin alone for 12 hours decreased the concentration of the basal labile iron pool compared to cells cultured in media only. Following subsequent exposure to iron, cells pre-loaded with quercetin demonstrated significantly decreased LIP levels over a 24 hour time period despite there being higher levels of total cellular iron (as assessed by radioisotope studies). These results further support the hypothesis that intracellular quercetin is binding iron imported into the cell rendering it unavailable and not contributing to the LIP. In addition, changes in TfR1, IRP2 and ferritin were also observed and the changes are consistent with this suppression in the LIP. The influence of either i) the localisation of quercetin or ii) the concentration of free quercetin in quercetin *and* iron supplemented media may rationalise why a 'shuttling' effect has been reported.[36]

Whether the anti-oxidant effects of quercetin are in part through its iron-modulatory effects is unclear,[20, 37] and a variety of experiments performed herein aimed to delineate this. Co-culture of cells with quercetin for 36 hours significantly decreases GPX4 (an anti-oxidant enzyme produced in response to ROS-generating potential) expression compared to cells co-cultured with iron only. This suggests two possible mechanisms i) that quercetin is having a direct anti-oxidant effect (i.e. directly sequestering ROS) or ii) that quercetin is chelating the endogenous iron within cells (which has been demonstrated by increased IRP2 and increased TfR1 protein expression) which is dampening iron-mediated ROS production. To delineate the two possibilities, cells were pre-loaded with iron for 12 hours or co-cultured in control growth media and then exposed to quercetin for 24 hours before assessment of GPX4 protein expression. Quercetin was able to reduce the ROS-generating potential of

iron within cells compared to those exposed to iron only, suggesting that quercetin acts to reduce ROS by directly chelating iron, disabling iron's ability to take part in redox reactions. This anti-oxidant effect was evident in the presence of sodium ascorbate, which is known to promote Fenton-mediated production of ROS in the presence of iron.[38] These results were also mirrored when direct levels of ROS were measured, where quercetin was able to reduce ROS concentration directly through the chelation of basal endogenous iron, exogenous iron within the media and the chelation of absorbed iron. It is clear that the action of quercetin as an anti-oxidant is directed through its iron chelating potential and within our cell studies, quercetin is targeting solely this free-iron as the media was supplemented with sodium ascorbate.

These studies demonstrate that quercetin can bind extracellular iron and the resultant quercetin-iron complex remains extracellular. However, in the absence of extracellular iron, quercetin is likely to become internalised and upon exposure to iron, forms an intracellular complex which remains intracellular. The intracellular quercetin-iron complex is not able to contribute to the labile iron pool and neither is it able to modulate ferritin, TfR1 and IRP2 expression as non-ligated iron normally would. Likewise, quercetin chelated iron is unable to participate in ROS-generating redox reactions, and this anti-oxidant ability of quercetin is attributed to its iron-chelation ability. A schematic mechanism of this model is outlined in Figure 7.

The polyphenol quercetin is as an ideal candidate as a dietary iron chelator and thus may possess anti-cancer activity. These data identify quercetin as an ideal agent to chelate free 'luminal iron' within the gastrointestinal tract, and, owing to its intracellular iron-modulating effects in broader applications throughout the body where intracellular iron sequestration is required. If quercetin were to be used as a therapeutic agent in the chelation of free-iron within the lumen of the colon, several pharmacological factors must be considered. This includes the effective dose of quercetin that would reach the colon at the concentration that we have observed efficacy (200 μM). Although bioavailability of quercetin is low, colonic

concentrations of quercetin have also been found to be low (0.63 µM) in subjects consuming average daily servings of fruits and vegetables.[27, 39] Such low levels would warrant the need for quercetin-supplementation to obtain the higher efficacious concentrations. Another factor to consider is the metabolism of quercetin by the colonic microbiome which has been documented.[40, 41] The metabolism of quercetin within the colon would reduce the effective concentrations present. Supplementing quercetin within colonic-delivery systems would deliver much higher doses of quercetin directly to the colon, allowing iron chelation to take place before considerable breakdown is observed.[42] Despite this, downstream metabolites of quercetin have also been reported to bind iron and many other polyphenolic compounds are metabolised into quercetin itself.[43, 44] In addition, whether iron-complexes of quercetin are metabolised similarly to native quercetin is unknown, yet many flavonoids have altered chemical properties and biological interactions when complexed with a metal which are distinct from the parent flavonoid.[45] A colonic delivery would also ensure that the quercetin does not interfere with iron absorption within the duodenum, minimising the risk of any subsequent iron deficiency developing.

In support of such a therapeutic approach by chelating free-iron within lumen of the colon, there have been murine studies highlighting quercetin as an anti-cancer agent. In one study, administration of a 0.2 % quercetin diet decreased total intestinal polyp formation by 67 % compared to a placebo cohort in a spontaneous murine model (ApcMin/+) of intestinal cancer.[46] In a separate study employing azoxymethane- induced rat colon cancer model, quercetin administration resulted in a four-fold suppression in aberrant crypt foci.[47] In light of results generated here, it could be envisaged that this anti-carcinogenic function maybe a result of iron-chelation, such that iron is unable to modulate oncogenic pathways such as Wnt and/or participate in Fenton type reactions in the generation of ROS.

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## AUTHORS CONTRIBUTION

RH and DH performed the experiments. RH, THI, and CT conceived and designed the experiments. RH and DH analysed the data. RH and CT wrote the paper.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## TABLES

**Table 1** Iron binding properties calculated by isothermal titration microcalorimetery under physiological conditions.

| Polyphenol | | Quercetin | Rutin |
|---|---|---|---|
| **Number of binding sites on polyphenol (N)** | Fe(II) | 1 | 2 |
| | Fe(III) | 1 | - |
| **Iron binding constant (K)** | Fe(II) | $8.3 \times 10^5\ M^{-1}$ | $3.2 \times 10^8\ M^{-1}$<br>$2.2 \times 10^4\ M^{-1}$ |
| | Fe(III) | $3.86 \times 10^6\ M^{-1}$ | - |

## FIGURE LEGENDS

**Figure 1:** Thermograms and corresponding isotherms for ferrous iron ($FeSO_4 \cdot 7H_2O$, 0.5 mM in aqueous HCl (0.1 M)) binding to **(A)** quercetin, **(B)** rutin, **(C)** cyanidin-3-O-glucoside and **(D)** catechin, and ferric iron ($FeCl_3 \cdot 6H_2O$, 0.5 mM in aqueous HCl (0.1 M)) binding to **(Ai)** quercetin, **(Bi)** rutin, **(Ci)** cyanidin-3-O-glucoside and **(Di)** catechin. The solid lines represent the curve fitting results using the model of best fit.

**Figure 2:** Ferritin protein expression in RKO cells co-cultured with iron ($FeSO_4 \cdot 7H_2O$, 100 μM) in the presence or absence of polyphenols (**(A)** quercetin, **(B)** rutin, **(C)** cyanidin-3-O-glucoside and **(D)** catechin) between 0 - 200 μM for 24 hours as assessed by Western blotting. **(E)** Representative Westerns blots used for densitometry. Data points represent mean fold change in protein expression normalised to β-actin, relative to iron only control. Error bars donate ± SEM with * indicating statistical significance with $p < 0.05$ and ◊ denoting $p < 0.001$, $n = 3$.

**Figure 3:** Intracellular $^{59}Fe$ concentrations in RKO cells treated with **(A)** quercetin and **(B)** rutin, at between 0 – 200 μM concentrations. * denotes statistical

significance ($p < 0.05$) vs. iron only control. ◊ denotes statistical significance $p < 0.001$. Error bars denote ± SEM, n = 12.

**Figure 4:** **(A)** Intracellular $^{59}Fe$ concentrations for RKO cells treated with quercetin (200 μM) or iron for 12 hours and after this time period, incubation in $^{59}Fe$ supplemented media ($FeSO_4 \cdot 7H_2O$, 100 μM + 10,000 $^{59}Fe$ CPM/mL media) for 24 hours. * denotes statistical significance ($p < 0.05$) vs. iron only controls. Error bars denote ± SEM, n = 8. **(B)** Changes in the LIP in RKO cells co-cultured with quercetin (200 μM) or media control. (C) Changes in the LIP in RKO cells pre-cultured with or without quercetin (200 μM) for 12 hours prior to co-culture with iron ($FeSO_4 \cdot 7H_2O$, 100 μM) for 24 hours. * denotes statistical significance ($p < 0.05$), error bars denote ± SEM, n = 8. Q = quercetin and M = Media only

**Figure 5:** **(A)** TfR1, **(B)** Ferritin, **(C)** IRP2 and **(D)** GPX4 expression in RKO cells co-cultured with or without quercetin (200 μM) for 12 hours prior to co-culture with iron ($FeSO_4 \cdot 7H_2O$, 100 μM) for 24 hours as assessed by Western blotting. **(E)** Representative Westerns blots used for densitometry Data points represent mean fold change in protein expression normalised to β-actin. Error bars denote ± SEM with * indicating statistical significance with $p < 0.05$, n = 3. Q = quercetin and M = Media only.

**Figure 6:** **(A)** Intracellular ROS concentrations in RKO cells co-cultured with quercetin (20 μM) for 3, 12 and 24 hours. **(B)** Intracellular ROS concentrations in RKO cells co-cultured with quercetin (20 μM) and iron ($FeSO_4 \cdot 7H_2O$, 100 μM) for 3, 12 and 24 hours. **(C)** Intracellular ROS concentrations in RKO cells when preloaded with iron ($FeSO_4 \cdot 7H_2O$, 100 μM) prior to co-culture with quercetin 3, 12 and 24 hours. * denotes statistical significance, $p < 0.05$. Error bars denote ± SEM, n = 12. **(D)** GPX4 protein expression in RKO cells co-cultured

with or without iron ($FeSO_4 \cdot 7H_2O$, 100 μM) for 12 hours prior to co-culture with quercetin (200 μM) or further iron for 24 hours. Data points represent mean fold change in protein expression normalised to β-actin, relative to iron only control. Q = quercetin and M = Media only

**Figure 7:** Schematic representation of the intracellular and extracellular actions of quercetin in the presence and absence of iron. (Fe = Iron, LIP = Labile Iron Pool, ROS= Reactive Oxygen Species).

FIGURE 1

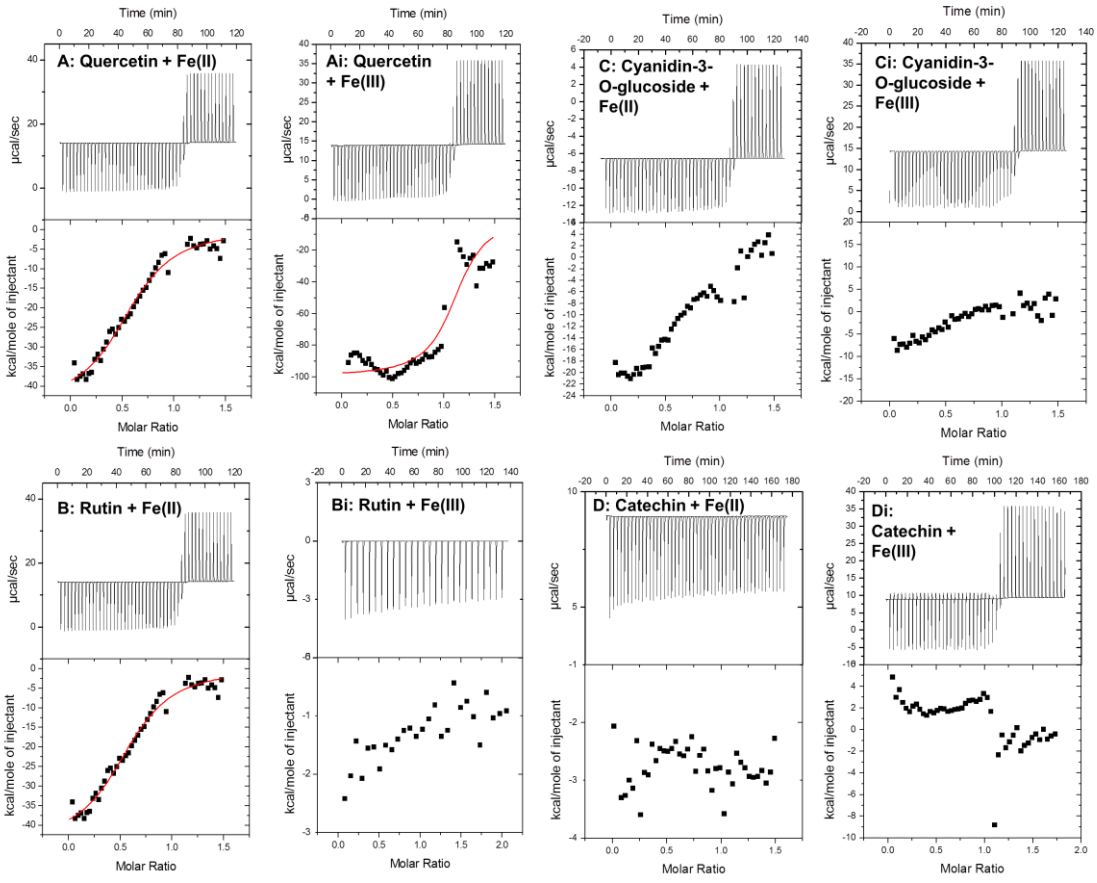

**FIGURE 2**

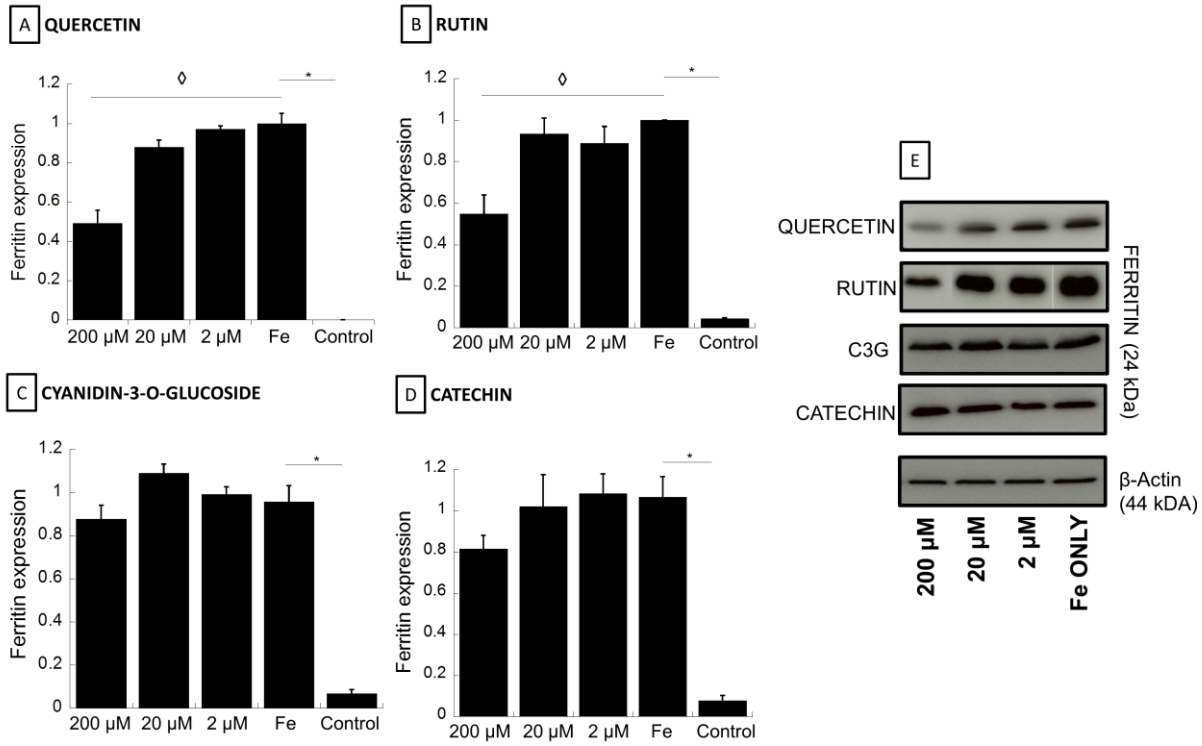

**FIGURE 3**

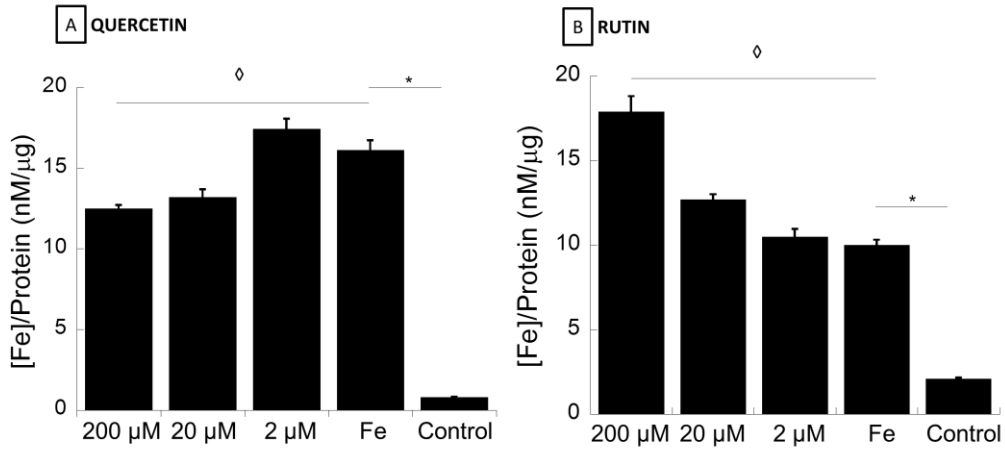

**FIGURE 4**

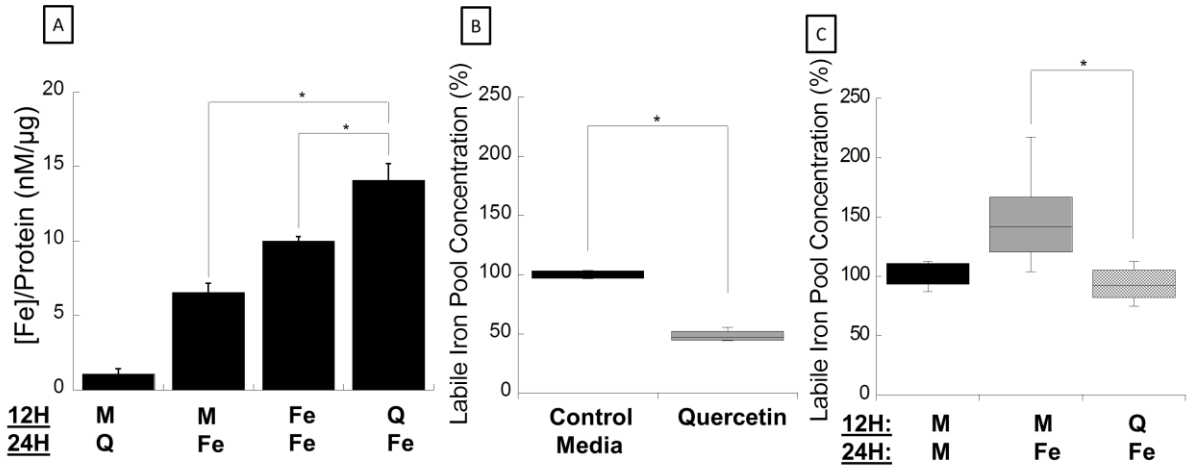

**FIGURE 5**

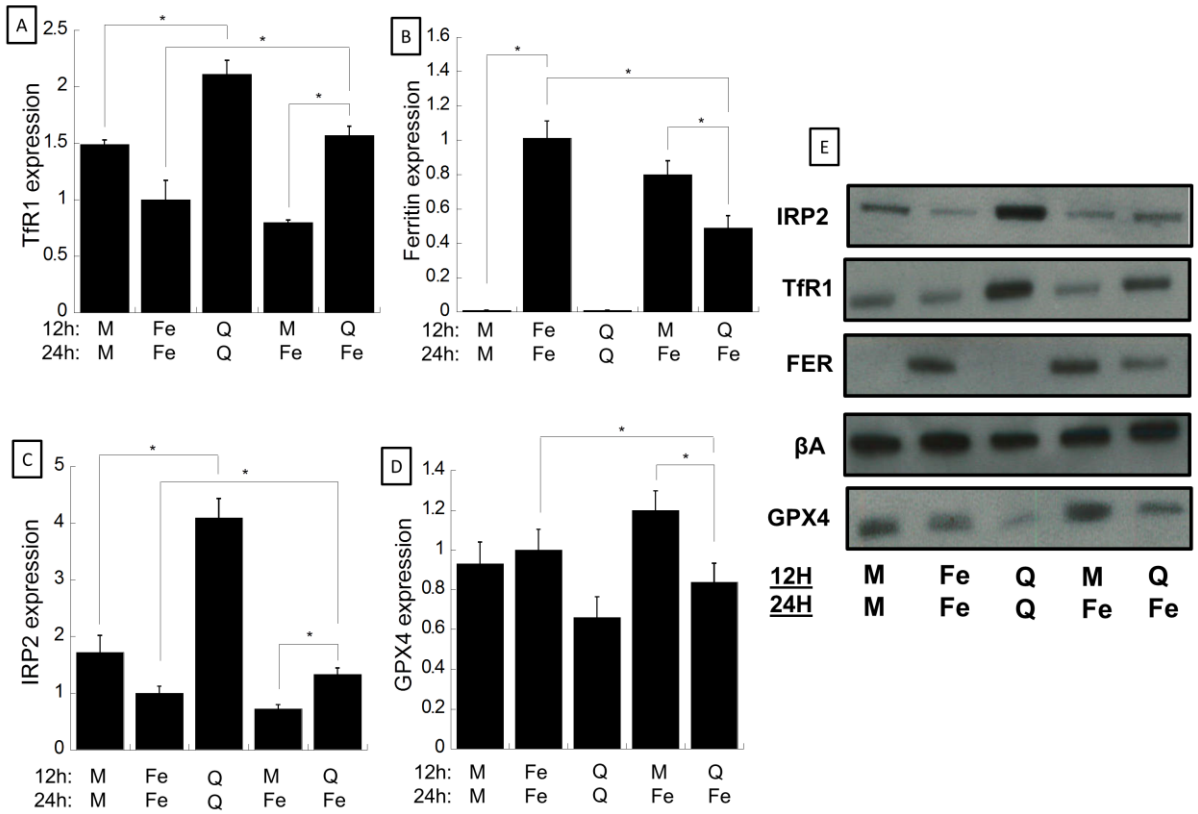

**FIGURE 6**

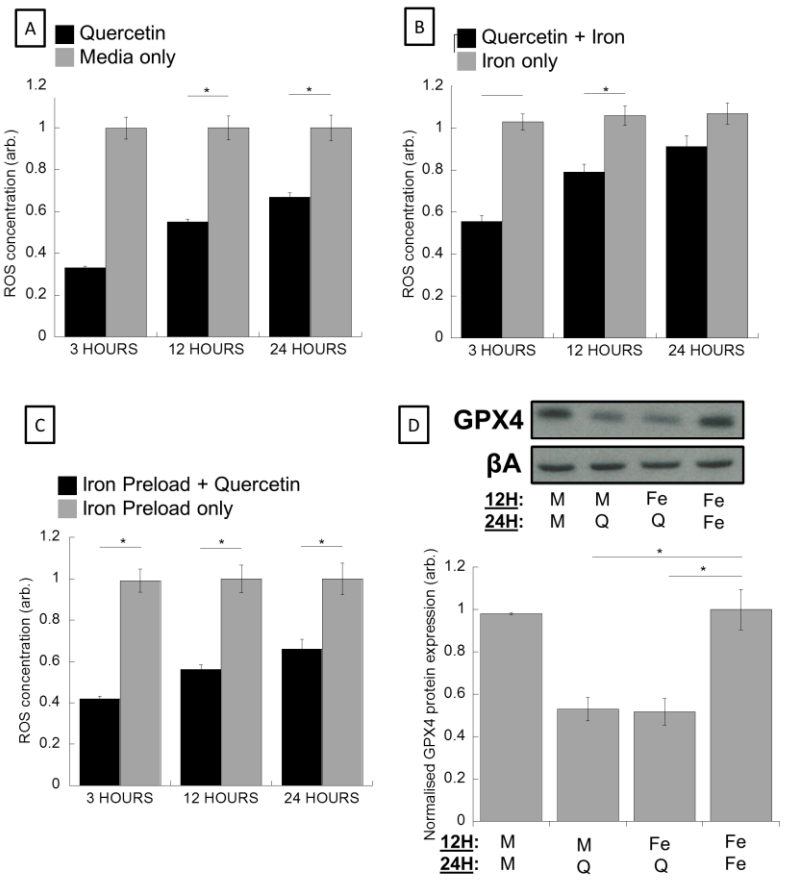

**FIGURE 7**